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The Hypocholesterolemic Effect of Green Tea EGCG Was Not Mediated Via the Stimulation of the Low-Density Lipoprotein Receptor Gene Expression in Cholesterol-Fed Rats*

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Green tea, which has high polyphenols amount, is thought to have hypocholesterolemic effects. The present study was performed to further examine the hypocholesterolemic action of green tea, especially (-) epigallocatechin gallate (EGCG) for its effect on diet-induced hypercholesterolemia in rats. Male Sprague-Dawley rats (n=15) were fed a green tea-free diet (control), 1.0% green tea catechin (catechin) or 0.5% green tea catechin EGCG for seven weeks. Hypercholesterolemia was induced by adding 1% cholesterol and 0.5% cholic acid to all diets. There was no difference in food intake and body weight gain among the groups. The green tea EGCG treatment led to a significant improvement in plasma levels of total cholesterol, low density lipoprotein (LDL)-cholesterol and high density lipoprotein (HDL)/LDL ratio ($p<0.05$). There was no significant effect on the plasma HDL-cholesterol level. The catechin treatment led to a 4.19-fold increase in the LDL-receptor mRNA level compared to the control, but the EGCG treatment did not affect the hepatic LDL-receptor mRNA level. Our results suggest that when blood cholesterol level is down-regulated by green tea EGCG, the LDL receptor gene-independent pathway may dominate the hypocholesterolemic action of EGCG.

Key word : Green tea, Catechin, EGCG, Hypocholesterolemic, LDL-receptor mRNA

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INTRODUCTION

An increased blood cholesterol level is one of the major risk factors for the development of cardiovascular disease (CVD). According to World Health Organization estimates, 17 million people around the globe die by CVD each year.[1] Blood cholesterol is taken through the low-density lipoprotein (LDL) receptor into the liver. A decrease of LDL receptors causes an increase in the circulating LDL-cholesterol level, leading to cardiovascular disease. Regulation of hepatic LDL receptor expression is thus of primary importance in controlling the blood cholesterol level. Up-regulation of the LDL receptor implicates enhanced clearance of blood cholesterol and further suggests a reduced risk of CVD.[2]

In recent years, accumulating evidence points to the role of certain dietary components in the prevention of high blood cholesterol. Among them, we are especially interested in the green tea catechins. Epidemiological studies in Japan have identified an inverse association between the consumption of green tea and the blood concentration of cholesterol,[3-5] a major risk factor in the development of CVD.[6] Consistent with this observation, numerous intervention studies in animal models have found that green tea or green tea extracts enriched in catechins exhibit a hypocholesterolemic effect.[7-12] Green tea extract treatment significantly lowered serum and liver cholesterol levels and increased HDL/LDL cholesterol ratios in rats fed a cholesterol-enriched diet.[9] Also, Chan *et al.*[12] reported that plasma lipid profiles improved significantly with the intake of the tea extract compared to the control. The control group received distilled water and the other two groups received either green tea water extract (GTWE) or green tea extract solution (GTE). Both the GTWE and GTE groups had 10% and 19% lower concentrations of serum total cholesterol, respectively, than the control group ($P<0.05$). All green tea extract treatments significantly improved serum levels of total cholesterol, triacylglycerols and apolipoprotein B in the following order: GTE>GTWE>Control ($P<0.05$).

The action mechanism for lowering the blood cholesterol level by green tea may involve enhanced clearance of

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cholesterol from the blood. Such increased clearance would likely be the result of increased expression of the LDL receptor, the major mechanism by which cholesterol is removed from the blood circulation.[13] Christina *et al.*[14] reported that LDL receptor binding activity and protein levels increased in HepG2 cells treated with EGCG. However, the effect of green tea EGCG on LDL receptor gene expression has not yet been investigated *in vivo*. In the present study, the effect of green tea EGCG on LDL receptor gene expression was investigated using animals in order to elucidate the hypocholesterolemic action of green tea.

MATERIALS AND METHODS

1. Experimental Animals and Diets

Male Sprague-Dawley rats (initial weight, 150±5 g, SLC, Japan) at four weeks of age were housed individually in a temperature- (22±2 ℃), relative humidity- (55±5%) and light- (dark, 06:00-18:00 h) controlled room. The rats were given free access to a non-purified diet (Rodent Laboratory Chow, Ralston Purina, St. Louis, MO) and tap water for one week in order to acclimatize them before the experiment. After one week of acclimatization, the rats were randomly divided into three groups (n=5) and assigned to different dietary treatments.

The compositions of the experimental diets are shown in Table 1. Mineral (AIN-76) and vitamin (AIN-76) mixes were purchased from Harlan Teklad (Madison, U.S.A). The rats were fed a green tea-free diet (control) or diets supplemented with 1% green tea catechin (Novanat, China) or 0.5% EGCG ad libitum for seven weeks. All green tea diets were prepared to provide approximately 0.5% EGCG. Because green tea catechin contains about 50% EGCG,[15] 1% catechin was added to provide 0.5% EGCG. The EGCG (85% purity) extracted from green tea catechin was kindly donated by TaePyongYang technology institute (Yongin, Korea) (Fig. 1). The analytical HPLC column was FALSH 40M column packed with Resin: HP-20 (4.0×15.0 cm). An isocratic mode was used and the mobile phase was 0.02% ascorbic acid in 15% ethanol at a flow rate of 25 μl/min.

Table 1. Compositions of experimental diets[1]

Component	Control	Catechin	EGCG
		(g/100g diet)	
Casein	20	20	20
D,L-methionine	0.3	0.3	0.3
Corn starch	13.5	12.5	13
Sucrose	45	45	45
Cellulose	5	5	5
Lard	10	10	10
Mineral mix(AIN-76)[2]	3.5	3.5	3.5
Vitamin mix(AIN-76)[3]	1	1	1
Choline bitartarate	0.2	0.2	0.2
Cholesterol	1	1	1
Cholic acid	0.5	0.5	0.5
Catechin	-	1.0	-
EGCG	-	-	0.5
Total	100	100	100

1) Diets were AIN-76 semipurified, and given in powdered form.
2) AIN-76(No.170915) mineral mixture were purchased from Harlan Teklad (Madison, U.S.A).
3) AIN-76(No.40077) vitamin mixture were purchased from Harlan Teklad (Madison, U.S.A).

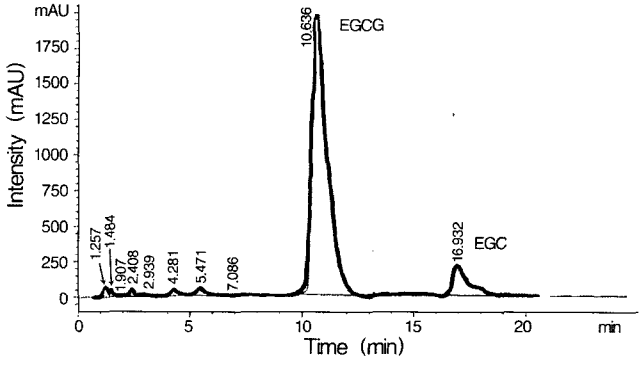

Fig. 1 Chromatogram of catechin compounds with different times. (HPLC analysis condition: 0.02% ascorbic acid in 15% ethanol, column: FALSH 40 M, flow rate: 25 μl/min, 20 μl injection)

All diets contained 1% cholesterol and 0.5% cholic acid in order to induce hypercholesterolemia. Food intake and body weight gain were monitored twice per week. At the end of the experiment, the rats were deprived of food for 16 hours and then anesthetized using dry ice. A central longitudinal incision was made into the abdominal wall and blood samples were collected by cardiac puncture with syringes containing 1 g EDTA/L blood. The blood samples were centrifuged at 1500×g for 20 min at 4 ℃ and the plasma was separated and stored at -20 ℃ until analyzed. Liver samples were excised, immediately frozen in liquid nitrogen and stored at -70 ℃ until analyzed. All animal procedures described conformed to NIH guidelines.[16]

2. Determination of Cholesterol and Triacylglyceride Concentrations in Plasma

Plasma total cholesterol, HDL-cholesterol and triacylglyceride levels were determined by enzymatic colorimetric methods using commercial kits (Sigma, St Louis, MO)

without extraction. Plasma LDL cholesterol was calculated using the formula of Friedewald *et al.*.[17]

Friedewald Calculation:

(LDL-C)=(Total Cholesterol)-(HDL)-(0.2×Triglycerides)

3. Real-time PCR Analysis

To assay for the LDL-receptor mRNA, total RNA was first isolated using the procedure of Chomczynski and Sacchi.[18] Liver tissue was placed in 10 mL of extraction solution D and manually homogenized on ice. Then, 1 mL of 2 M Na acetate, 10 mL of Phenol and 2 mL of Chloroform: IAA (49:1) was added to the homogenate liver tissue. The aqueous and organic phases were separated using a centrifuge at 4,500 rpm for 30 min at 4 ℃. The aqueous layer was transferred to a Corex tube. Then, 10 mL of ethanol was added and the solution was stored at -70 ℃ for overnight. The ethanol was removed and stored on ice for 20 minutes. Then, 800 μl of solution D was added and the solution was stored at -20 ℃ for 1 hour. It was centrifuged at 4,500 rpm for 20 min at 4 ℃ before the supernatant was removed. The pellets were washed in 70% ethanol and centrifuged again. The supernatant was discarded and the precipitate was dried in the air. The precipitate was then dissolved in 40 μl of DEPC- water. RNA concentration was quantified using a UV spectrophotometer at 260 nm and the purity was determined by A_{260}/A_{280} ratio. All samples were reverse-transcribed using moloney-murine leukemia virus reverse transcriptase (Promega, U.S.A) and oligo dT_{19} (30 mole) in 20 μl of total reaction volume containing 5X RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) and 1 mM dNTPs. Real-time PCR assay was performed to specifically quantify the mRNA level. In all assays, cDNA was amplified using a standardized program (10 min denaturing step; 55 cycles of 5" at 95 ℃, 20" at 60 ℃, and 20" at 72 ℃: melting point analysis in 1 ℃ step: final cooling step) using Rotor-Gene 3000 (Corbett Research, Australia). The primers used for β-actin were forward; 5'-GGA CCT GAC AGA CTA CCT CA-3', reverse; 5'-GTT GCC AAT AGT GAT GAC CT-3', for LDL-receptor were forward; 5'-CAA GGA GTC CAA GAC CAA CGA-3' reverse; 5'-TGG GAA CAG CCA CCA TTG T-3'. The delta delta C_T method was used to measure relative quantification. First, the delta C_T value for each sample was determined by calculating the difference between the C_T value of the LDL-receptor target gene and the C_T value of the beta-actin references gene. This was determined for each unknown sample as well as for the calibrator sample. Next, the delta delta C_T value for each sample wss determined by subtracting the C_T value of the calibrator from the C_T value of the sample. The normalized LDL-receptor target gene expression level in sample was calculated by using the formula, $2^{-\Delta\Delta Ct}$.

4. Statistical Analysis

Data are expressed mean±SE. Data for the control, catechin and EGCG groups were analyzed by one-way ANOVA; $P \geq 0.05$ was taken as indicating no significant difference. Where ANOVA showed significance, the differences among groups were evaluated using Duncan's multiple range test.[19]

RESULTS

1. Body Weight Gain, Food Intake and Food Efficiency

Random assignment of the rats to three experimental groups did not result in any differences in initial body weight (Table 2). There was no difference in rates of body weight gain among the groups fed the control, 1% catechin or 0.5% EGCG diets. Also, food efficiency was not affected by catechin and EGCG.

Table 2. Body weight gain, food intake, and food efficiency of rats fed experimental diets[1)]

Group	Initial body weight (g)	Weight gain (g/daily)	Food intake (g/daily)	Food efficiency (Weight gain/ food intake)
Control	124.4±13.1^{NS}	6.40±0.69^{NS}	22.38±0.53^{NS}	0.29±1.3^{NS}
Catechin	121.1±16.9	7.03±0.81	22.69±0.52	0.30±1.6
EGCG	128.1±15.3	6.30±0.7	20.91±0.35	0.30±2.0

1) Values are expressed as mean±SE, n=5
NS is not significant

2. Effect of Green Tea on Plasma Lipid Profiles

Rats treated with 1% catechin or 0.5% EGCG showed significantly lower plasma total cholesterol levels of 14% and 27%, respectively, than the control group ($P<0.05$) (Table 3). The LDL-cholesterol concentration was significantly lower in the EGCG-supplemented group (up to 25%) than in the control group ($P<0.05$). Although green tea supplementation had no significant effect on HDL-cholesterol and triglyceride concentrations in plasma, the HDL/LDL ratio rose 80% compared to the figure for the control group ($P<0.05$).

3. Effect of Green Tea on the LDL Receptor mRNA

Catechins, present in high amounts in green tea, have been proposed as the constituents that lower plasma cholesterol levels in animals. Rats fed a diet supplement with green tea catechins showed a 4.19-fold elevation in LDL-receptor mRNA compared to rats fed a control diet (Fig. 2). Feeding with an EGCG-supplemented diet resulted in a slight increase in LDL-receptor mRNA compared to the control group. But the EGCG diet had no significant effect on LDL-receptor gene expression.

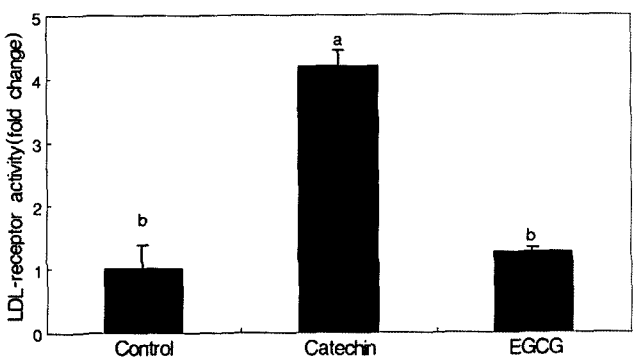

Fig. 2 LDL-receptor mRNA expression in liver of rats fed experimental diets.

Data were processed with a specially designed software program based on Ct values of each sample and normalized to ß-actin mRNA (n=3). Values in bars with different superscripts are significantly different ($p<0.05$) and expressed as mean±SE, n=3

DISCUSSION

In this study, we added 1% catechin contributing approximately 0.5% EGCG or 0.5% EGCG to diets containing 1% cholesterol and 0.5% cholic acid for seven weeks. There was no difference in the rates of body weight gain among rats fed the control, 1% catechin or 0.5% EGCG diets. Yang *et al.*[9] induced hypercholesterolemia with 1% cholesterol and 0.5% cholic acid and treated their test subject rats with 2% green tea for eight weeks. Similar to our results, they observed no significant difference in body weight among the control and green tea-supplemented groups. Also, Chan *et al.*[12] reported that no significant differences in body weight gain or food intake were observed among the control, green tea water extract and green tea extract solution groups. These results suggest that green tea did not affect food intake or body weight gain in animals fed a normal-energy diet.

Green tea catechin consists mainly of four derivatives, including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG). Animals fed a green tea EGCG-supplemented diet showed a significant decrease of up to 25% in blood LDL-cholesterol concentrations compared to the control group ($P<0.05$) (Table 3). Animals fed a green tea catechin-supplemented diet showed lower blood LDL-cholesterol concentrations of 16% compared to the control group, though this result was not significant. Our results indicate that EGCG in green tea catechin may be the major active component or contribute at least in part to the hypocholesterolemic effect of green tea.

The HDL/LDL ratio increased significantly in rats fed an EGCG-supplemented diet compared to those fed a control diet. The increase in the HDL/LDL ratio could be due to a significant decrease in the plasma LDL-cholesterol level and a slight increase in the HDL-cholesterol level, which implies an antiatherogenic effect. But, the EGCG diet had no significant effect on plasma triglyceride concentrations. Daniel *et al.*[20] fed rats a diet high in cholesterol and fat containing 0, 0.25, 0.5 or 1.0% of EGCG for four weeks. Blood triglyceride and HDL-cholesterol levels were unchanged by the EGCG treatment, similar to the results of the present study.

The major pathway of blood cholesterol clearance is through the LDL-receptor. Increased cholesterol clearance would thus most likely be the result of increased expression of the LDL-receptor, the major mechanism by which cholesterol is removed from the blood circulation. Thus, it can be hypothesized that EGCG may

Table 3. Lipoprotein cholesterol and triglyceride concentrations in plasma of rats fed experimental diets[1,2]

Group	Total cholesterol	LDL cholesterol[3]	HDL cholesterol	Triglyceride	HDL/LDL
	(mg/dl)				
Control	221.1±8.7^{a}	210.2±0.80^{a}	10.8±0.7^{NS}	60.5±0.09^{NS}	0.05±0.06^{b}
Catechin	189.3±13^{ab}	177.1±0.39^{ab}	12.0±0.07	52.7±0.07	0.07±0.17^{ab}
EGCG	161.5±10.9^{b}	157.8±0.22^{b}	13.3±0.19	48.2±0.15	0.09±0.16^{a}

1) Values are expressed as mean±SE, n=5
2) Values in a column with different superscripts are significantly different, $p<0.05$
3) LDL cholesterol was calculated by the method of Friedewald WT formula
NS is not significant

induce LDL receptor gene expression for its hypocholesterolemic action. In this study, the effect of cholesterol clearance in rats fed green tea was to be expected due to high LDL receptor gene expression. The EGCG diet resulted in a 1.2-fold increase in LDL receptor mRNA compared to the result for the control group, but this was not significant (Fig. 2). Moreover, a catechin diet led to a 4.19-fold increase in LDL receptor mRNA. The fact that the level of LDL receptor gene expression was highest in rats fed catechin may be the result of the fact that catechin correlates not only with EGCG but also builds up complexes of EGC, ECG and EG. These results suggest that the hypocholesterolemic effect of EGCG may not be mediated through LDL receptor gene expression.

Recently, it was reported that isoflavones are beneficial in the lowering of serum cholesterol and non-HDL cholesterol, but that isoflavone supplementation has no significant effect on the level of the hepatic LDL receptor mRNA.[21]

Daniel *et al.*[20] reported that green tea catechin EGCG reduced cholesterol absorption from the intestine by reducing the solubility of cholesterol in mixed micelles. They suggested that one of the underlying mechanisms by which EGCG affects cholesterol metabolism is by interfering with the micellar solubilization of cholesterol in the digestive tract, which in turn decreases cholesterol absorption and lowers blood cholesterol. Also, several animal studies have shown that blood cholesterol was reduced as a result of the fact that catechin inhibited cholesterol absorption.[22-23] Chisaka *et al.*[24] showed that EGCG did not inhibit cholesterol synthesis in rats but orally administered EGCG decreased cholesterol absorption from the intestine. Ikeda *et al.*[25] reported that tea catechins inhibited lymphatic absorption of cholesterol.

Another mechanism that may explain the hypocholesterolemic effect of EGCG is that EGCG may enhance cholesterol 7α-hydroxylase 1 (CYP7A1) gene expression, resulting in increased fecal excretion of bile acids. Conversion of cholesterol to bile acids is the major pathway of cholesterol elimination. CYP7A1, the rate-limiting enzyme in the conversion of cholesterol to bile acids, is mainly regulated by the feedback inhibition of bile acids re-absorbed from the intestine. This mechanism, supported by the findings of Yang et al.,[22] showing that 4% Chinese Lung Chen tea increased CYP7A1 activity with significantly increased fecal cholesterol and bile acids excretion. They suggested that the hypocholesterolemic effects of green tea diet might be due to the enhancement of CYP7A1 gene expression resulting in the increased fecal excretion of bile acids.

In conclusion, EGCG showed a hypocholesterolemic effect in rats in which hypercholesterolemia was induced with cholesterol. The hypocholesterolemic effect of green tea EGCG was not mediated via the stimulation of LDL receptor gene expression. Therefore, further study should be carried out to clarify the exact mechanism behind the hypocholesterolemic action of EGCG.

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